Medical and Pediatric Oncology 27:453–455 (1996)

Functional Properties of *WT1*

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WT1 encodes a zinc finger transcription factor that is inactivated in a subset of Wilms' tumors. We have recently shown that introduction of wild-type *WT1* into a Wilms' tumor-derived cell line, *RM1*, results in growth suppression, consistent with its function as a tumor suppressor gene. *WT1*-mediated growth suppression was also observed in other cells derived from embryonal tumors, including two osteosarcoma cell lines, U2OS and Saos-2, notable for the respective presence or absence of wild-type p53. To further characterize the functional properties of *WT1*, multiple U2OS and Saos-2 cell lines were established, expressing either wild-type *WT1* splicing variants or naturally occurring mutants under control of a tightly regulated tetracycline repressable promoter. Induction of *WT1* in these cells resulted in programmed cell death. This effect was preferentially mediated by *WT1* isoform B (encoding alternative splice I, lacking alternative splice II "KTS"), and it was independent of p53, occurring in both U2OS and Saos-2 cells. *WT1*-mediated apoptosis was associated with transcriptional repression of the epidermal growth factor receptor (EGFR) and reduced synthesis of endogenous EGFR protein synthesis. Constitutive expression of EGFR abrogated *WT1*-mediated cell death. We conclude that wild-type *WT1* can induce apoptosis in embryonal cancer cells, presumably through the withdrawal of required growth factor survival signals, and that EGFR is a physiological target gene for *WT1*.

Key words: Wilms' tumor, *WT1*, apoptosis, alternative splicing

INTRODUCTION

WT1 encodes a transcription factor that is developmentally regulated during kidney development and inactivated by mutation in a subset of Wilms' tumors. The C terminus of *WT1* contains four zinc finger domains that recognize a guanidine-cytidine (GC)-rich "EGR1" consensus sequence. The N terminus, rich in prolines and glutamines, appears to mediate transcriptional repression in transient transfection assays. A number of *WT1*-responsive promoters have been identified, based on the presence of GC-rich sequences: in transient transfection assays using promoter-reporter constructs, these promoters are transcriptionally repressed by cotransfection of *WT1* constructs. However, regulation of endogenous genes by *WT1* has not been demonstrated.

DISCUSSION

To study the functional properties of *WT1* and identify physiologically relevant *WT1* target genes, we first characterized cell lines in which *WT1* expression resulted in a clearly defined phenotype. We initially used a novel Wilms' tumor-derived cell line, RM1, to demonstrate the effect of *WT1* on cellular growth. RM1 cells are derived from an anaplastic Wilms' tumor and have the unique property of indefinite growth in culture, while retaining the ability to induce tumors following inoculation into nude mice. These tumors have a histologic appearance that is comparable to that of the original RM1 tumor, suggesting that the cultured cells have retained the potential for some degree of embryonal differentiation. The primary RM1 tumor and the derived cell line express low levels of *WT1* transcript, detectable by reverse transcription polymerase chain reaction (RT-PCR) analysis. This transcript, however, is remarkable for the in-frame deletion of exon 2, within the transcriptional repressor domain of *WT1* [1]. The deletion of *WT1* exon 2 does not appear to result from a mutation in any exon or flanking intron. We therefore presume that it is a consequence of aberrant *WT1* mRNA splicing in this tumor, a conclusion that is supported by the aberrant splicing of other alternatively inserted sequences within *WT1* (exon 5; see below), but the appropriate alternative splicing of unrelated genes such as CD44. Analysis of normal human and mouse tissues expressing *WT1* did not reveal any evidence of an exon 2-deleted-*WT1* transcript (*WT1*del2). However, approximately 10% of sporadic Wilms' tumor specimens expressed this aberrantly spliced *WT1* mRNA at levels

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Received June 6, 1995; accepted February 6, 1996.

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of 50% or above the wild-type transcript, and most tumors contained low levels (1–5%) of *WT1*del2 mRNA. To test the functional properties of *WT1*del2, we engineered this altered *WT1* cDNA into a cytomegalovirus (CMV)-driven expression construct, and examined the transactivational properties of the encoded protein. Whereas wild-type *WT1* repressed transcription from a target GC-rich promoter, *WT1*del2 was a potent activator of this promoter. Thus, *WT1*del2 encodes an altered protein which may act to antagonize the function of wild-type *WT1*, enhancing the expression of potential target genes repressed by the wild-type gene product. We cannot predict the expression level of *WT1*del2 in Wilms' tumors at which its altered transactivational properties may become functionally significant. However, it presents a novel, nonmutational mechanism that may contribute to *WT1* inactivation in a significant subset of Wilms' tumors.

The presence of an aberrant *WT1* transcript in RM1 cells made it possible to test the effect of reintroducing the wild-type allele into these cells. Wild-type *WT1* itself is encoded by four alternative splicing variants, resulting from the variable insertion of two sequences: alternative splice I results from the insertion of exon 5, between the N terminus transactivation domain and the C terminus DNA binding domain. This insertion results in a moderate but consistent increase in transactivation by *WT1*. Alternative splice II (also known as KTS) results from the use of an alternative splice donor site between exons 9 and 10 of *WT1*, resulting in the insertion of three amino acids (lysine, threonine and serine) between zinc fingers 3 and 4. This insertion either abolishes or reduces *WT1's* recognition of potential DNA binding sites.

All four *WT1* isoforms are coexpressed in tissues expressing *WT1* [2]. We have defined these as: A, neither alternative splice present; B, + alternative splice I and − alternative splice II; C, − alternative splice I and + alternative splice II; D, both alternative splices present. Remarkably, the relative ratios of these *WT1* variants, as determined by RNase protection analysis is preserved during renal development, between normal fetal kidney and Wilms' tumors, and across species. The most prevalent isoform, D, accounts for 60–70% of *WT1* transcript, with isoforms B and C accounting for 10–20% each, and isoform A 5–10%. To test the functional properties of each *WT1* isoform, we transfected them by lipofection into RM1 cells, together with a linked G418 resistance marker. Transfection of any *WT1* isoform, alone or in combination, dramatically reduced the number of drug-resistant RM1 cells, consistent with a growth suppressive effect [1]. Mutant *WT1* constructs displayed no growth-suppressive effect. The few *WT1*-transfectants that did emerge expressed the transfected *WT1* allele, that remained wild-type, but produced undetectable amounts of *WT1* protein. Inoculation of these RM1 tranfectants into nude mice demonstrated a reduced and delayed tumorigenicity, compared with mock-transfected clones. We therefore concluded that wild-type *WT1* suppressed the growth of Wilms' tumor-derived cells, both in culture and in vivo.

While Wilms' tumor cells are appropriate to demonstrate the growth suppressing properties of *WT1*, they are not well-suited to define the mechanism underlying this effect. RM1 cells are aneuploid, poorly transfectable and harbor mutated p53 alleles, complicating analysis of transactivation by *WT1*. We therefore screened additional cell lines derived from embryonal tumors, and observed that osteosarcoma cell lines also displayed inhibition of growth by *WT1*. We characterized two cell lines, U2OS and Saos-2, that are highly transfectable, diploid and have been extensively studied for their relative presence (U2OS) or absence (Saos-2) of p53. In both U2OS and Saos-2 cells, *WT1* isoform B (+ alternative splice I, − alternative splice II) displayed dramatic growth-suppressive properties, compared to other *WT1* isoforms. To study the mechanism underlying this effect, we established U2OS and Saos-2 cell lines with a tightly regulated, tetracycline-repressable promoter, driving either *WT1* isoform B, isoform D, or mutant WTAR. Induction of *WT1* isoform D or WTAR had minimal effect on the growth of osteosarcoma cells. However, induction of *WT1* isoform B resulted in the induction of apoptosis [3]. Cell death occurred 3–7 days after expression of isoform B, and was independent of p53, occurring in both U2OS and Saos-2 cells. The delayed onset of apoptosis was consistent with transcriptional repression of a gene encoding a protein with slow turnover. However, an initial screening of genes containing previously postulated *WT1* target promoters failed to show any effect of *WT1* induction on endogenous gene products. One growth factor receptor, the epidermal growth factor receptor (EGFR) gene, however, showed dramatic reduction in new protein synthesis following induction of *WT1* isoform B expression. This was associated with transcriptional repression of the EGFR promoter within 24 hrs of *WT1* induction. EGFR is thus the first endogenous gene shown to be a physiologic target of *WT1*. To test whether EGFR suppression by *WT1* expression was a trigger for *WT1*-induced apoptosis, we transfected CMV-driven EGFR cDNA constructs into U2OS cells and isolated multiple clones, constitutively expressing EGFR. Constitutive expression of EGFR abrogated the ability of *WT1* to induce apoptosis. EGFR expression was not associated with an increased growth rate of cells in the absence of *WT1* induction, indicating that its anti-apoptotic effect resulted from prevention of *WT1*-mediated cell death, rather than a compensating increase in cellular growth rate.

The identification of EGFR as a physiologic *WT1* target whose repression contributes to *WT1*-mediated apoptosis has potential implications for both normal kidney development and Wilms' tumorigenesis. In the developing rat kidney, we found that EGFR expression pre-

cedes that of *WT1*, both in terms of the time of peak expression of these two genes and the developmental structures expressing them. *WT1* may therefore contribute to the regulation of EGFR in the developing kidney. A number of studies by other investigators have suggested a potential role for EGF, or its fetal counterpart TGF-α, as a survival factor in the developing kidney. Thus, suppression of EGFR by *WT1* may be a developmental trigger, inducing effective growth factor withdrawal in renal stem cells and directing the fate of these cells between differentiation and apoptosis. Further experiments involving differentiating kidney rudiments in vitro will be required to test this hypothesis.

The induction of apoptosis by *WT1* may also underlie its function as a tumor suppressor gene. We have previously shown that *WT1* mutations are present within nephrogenic rests, preneoplastic precursors of Wilms' tumor [4]. Inactivation of *WT1* in renal stem cells may therefore result in their immortalization. Nephrogenic rests may thus represent a population of persistent blastemal cells, providing an expanded pool of target cells for subsequent mutational events, leading to Wilms' tumor. This model would be consistent with the identification of *WT1* mutation as an initial, rate-limiting, or enabling event in the genesis of Wilms' tumor. Further work will be required to identify the additional genetic changes required to convert immortalized blastemal cells in nephrogenic rests to frankly malignant Wilms' tumor.

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COMMENTARY

Haber et al. selected osteosarcoma cell lines (rather than Wilms' tumor cell lines) to study the natural functions of *WT1*. The investigators specifically selected these cell lines because of the clear phenotype (growth inhibition) associated with expression of the *WT1* gene. Of note, studies on the functional properties of the *WT1* protein are somewhat complicated by the fact that the *WT1* protein is encoded by four distinct *WT1* RNAs (four so called isoforms). Haber et al. demonstrate that one of the four *WT1* isoforms (*WT1* isoform B) displays much greater growth-suppressive properties than the other three. They then go on to show that induction of *WT1* isoform B results in induction of apoptosis ("natural cell death") within 3–7 days, through repression of the epidermal growth factor receptor (EGFR) gene. As such, EGFR is the first endogenous gene shown to be a physiologic target for *WT1*. This relation has potential implications for both normal kidney development and Wilms' tumorigenesis.